Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus

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PNAS 1952;38;747-752
doi:10.1073/pnas.38.8.747

(1) The coefficients of the rth *derivatives for* $r \leq m$ *are* $0(1/r^{2m-r+\epsilon})$ *for some* $\epsilon > 0$.

(2) For $r > m$, $j \leq r - m$, *the* jth *derivatives of the coefficients of the* rth *derivative terms in* A *are* $0(1/r^{2m+j-r+\epsilon})$ *for some* $\epsilon > 0$.

Then if the Dirichlet problem for K *on* D *with zero boundary values has only the zero solution, the Dirichlet problem for* K *on* D *has a solution for every boundary value function* g *permissible with respect to* L.

[1] Browder, F. E., "The Dirichlet Problem for Linear Elliptic Equations of Arbitrary Even Order with Variable Coefficients," PROC. NATL. ACAD. SCI., **38**, 230–235 (1952).

[2] The definition of positivity used here is stronger than the usual form in $L^2(D)$.

[3] Courant-Hilbert, *Methoden der Mathematischen Physik*, Vol. II, Berlin, 1937; p. 489.

[4] Condition (*b*) for ellipticity of our previous note is superfluous since every elliptic differential operator can be written as the sum of a positive operator and one of lower order. This fact was observed by L. Gårding who has announced the semi-boundedness of the homogeneous operator L defined in the proof of Theorem 1 as well as results on the Dirichlet problem for equations with infinitely differentiable coefficients similar to those of our previous note; *Compt. rend.*, **253**, 1554–1556 (1951).

[5] The results of (*b*) contain similar results obtained for the general second-order equation by Carleman, "Uber die asymptotische Verteilung der Eigenwerte partieller Differentialgleichungen," *Ber. Sachs, Akad.*, **88**, 128–129 (1936), Satz V.

[6] John, F., "General Properties of Solutions of Linear Elliptic Partial Differential Equations," Proceedings of the Symposium on Spectral Theory and Differential Problems, Stillwater, Okla. (1951), Chap. III.

[7] Cf. reference 1, p. 232.

[8] Bergman, S., "The Kernel Function and Conformal Mapping," Mathematical Survey Series, 1950.

[9] Courant-Hilbert, Vol. II, p. 511.

[10] A sufficient condition for g to be permissible with respect to L is that $|(f, g)| \leq K\|f\|_m$ for all $f \in C_c^{2m}(D)$.

PRODUCTION OF PLAQUES IN MONOLAYER TISSUE CULTURES BY SINGLE PARTICLES OF AN ANIMAL VIRUS

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Read before the Academy, April 29, 1952

Research on the growth characteristics and genetic properties of animal viruses has stood greatly in need of improved quantitative techniques, such as those used in the related field of bacteriophage studies.

The requirements for a quantitative virus technique are as follows: (1) The use of a uniform type of host cell; (2) an accurate assay technique; (3) the isolation of the progeny of a single virus particle; and (4) the separate isolation of each of the virus particles produced by a single infected

cell. In bacterial virus work, production of plaques by single virus particles on a uniform bacterial layer fulfills the first three requirements. The fourth requirement is easily met, in the case of bacteriophages.

In this article we shall show that plaques can similarly be produced by animal viruses and that their properties fulfill the first three requirements.

We have found that the virus of Western Equine Encephalomyelitis,

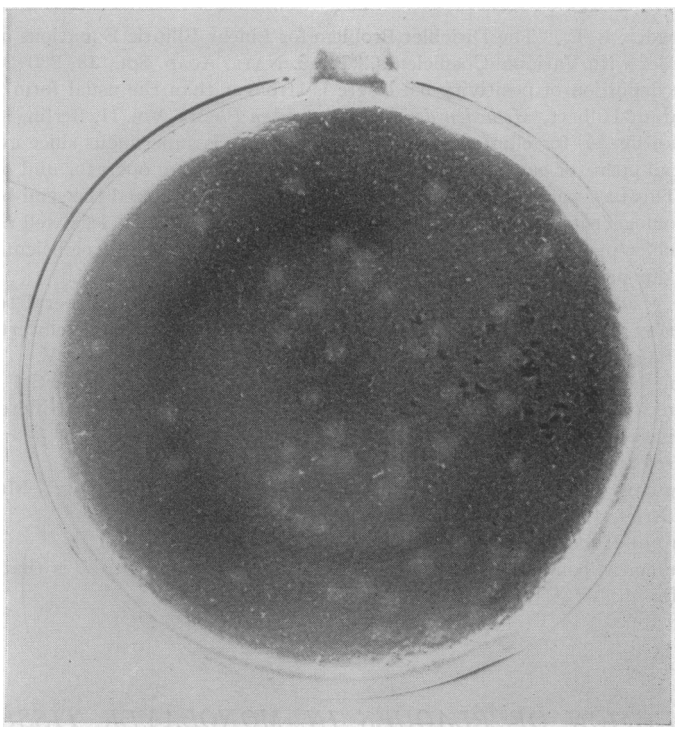

FIGURE 1

Plaques of Western Equine Encephalomyelitis virus on chicken fibroblasts. The plaques appear as round clear areas. The picture was taken against a distant dark background, sufficiently large to exactly cover the projection of the flask, surrounded by a uniformly brilliant light source, so as to detect the light scattered through a small angle by the cell debris in the plaques.

adapted to chicken embryo,[1] will produce plaques when it is grown on a monolayer of cells obtainable from chicken embryos by a modification of a technique devised by Shannon, Earle and Walts.[2] We use the following procedure: Under aseptic conditions, five 9-day-old chicken embryos are collected, decapitated, washed in Earle's saline solution (ES)[3] and pressed through a stainless steel, 24-mesh, wire cloth (supplied by Ludlow-Saylor

Wire Company, Los Angeles) which had been fitted at the bottom of a 50-ml. syringe. The resulting tissue pulp is collected in a 40-ml. centrifuge tube containing 15 ml. ES. After gravity sedimentation (10 minutes) the supernatant liquid containing cell debris and red blood cells is discarded and replaced with 15 ml. of a 0.5% solution of pure trypsin in ES. The tube is placed in a water bath at 37°C. for 10 minutes and then vigorously stirred by repeated pipetting with a 2-ml. automatic pipette. Owing to digestion of the connective fibers, a large fraction of the cells become free. The pieces of tissue which remain are eliminated by straining the suspension through the sieve unit described by Evans *et al.*[4] The cell suspension is then washed twice by centrifuging at 1000 r. p. m. for two minutes and resuspending the sediment in 15 ml. ES. The cell concentration in the final suspension is determined by counting in a hemocytometer.

An aliquot of the cell suspension containing 3×10^7 cells is introduced

TABLE 1

RELATIVE NUMBER OF PLAQUES PRODUCED BY WESTERN EQUINE ENCEPHALOMYELITIS VIRUS EXPOSED TO SPECIFIC ANTISERUM AND OTHER SUBSTANCES BEFORE INOCULATION INTO THE FLASKS

TIME OF CONTACT WITH THE VIRUS, MIN.	TREATMENT				
	DIST. WATER	ES	NORMAL HORSE SERUM 1:10 IN ES	HORSE SERUM HEATED AT 56°C. 30 MIN., 1:10 IN ES	HYPERIMMUNE HORSE SERUM HEATED AT 56°C. 30 MIN., 1:10 IN ES
0	1	1	1	1	1
7	..	..	..	0.92	0.15
10	..	..	0.63	..	..
15	1.1	0.8	..	0.74	0.085
20	..	..	0.25	..	..
22	..	..	..	0.67	0.055
30	0.9	1	..	..	0.01

into a large Pyrex Carrel flask (80 mm. inside diameter) which had been acid cleaned by the procedure described by Earle.[5] The volume is brought up to 5 ml. with ES. There are added 5 ml. of horse serum and 1 ml. of 1:1 chicken embryo extract. The flask is hermetically closed with a rubber stopper. Approximately two such flasks can be prepared from one embryo.

The flasks are incubated in horizontal position at 37°C.

During the first few hours of incubation the cells settle out and stick to the bottom of the flask singly or in small clumps. They start to grow very soon and within 48 hours produce a continuous cell layer which covers the bottom of the flask. The layer is mostly one cell thick and is formed by rather uniform spindle cells and by a small number of macrophages. The spindle cells are similar in morphology and rate of growth to the fibroblasts obtained by culturing the connective tissue of the chicken embryo; for this reason we called them fibroblasts. Other cell types carried with the

inoculum must obviously be present in the culture, but they are outnumbered by the fast-growing fibroblasts. A more uniform cell population can be obtained by using as inoculum a cell suspension obtained from the cell layer of one of these flasks by means of the described trypsin technique.

The relatively uniform cell layer is infected with the virus in the following way. The nutrient fluid is removed from the flask and the cell layer is washed with 5 ml. ES, which is then discarded. A virus sample of 0.5 ml. is introduced into the flask and uniformly spread on the cell layer by rocking. The flask is incubated for 30 minutes at 37°C. to allow attachment of of the virus to the cells, and the cell layer is then covered with a layer of

TABLE 2

NUMBER OF PLAQUES OBTAINED FROM STOCKS OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS AT DIFFERENT DILUTIONS

Experiments 1 and 2 were made with the same virus stock, experiments 3, 4 and 5 with a different stock. The virus consisted always of 20% extract of chicken embryo moribund after inoculation with the virus. Each flask was inoculated with 0.1 ml. of the diluted virus.

NUMBER OF THE EXPERIMENT	DILUTION OF THE VIRUS	NUMBER OF PLAQUES ON EACH FLASK
1	5×10^3	117; 180
	10^4	65; 87
2	10^4	59; 53
	3×10^4	28; 13; 18; 14; 19; 14; 18
3	10^3	79
	3×10^3	23
4	10^3	50
	4×10^3	12
	8×10^3	2
5	2×10^3	26
	6×10^3	10

5 ml. of melted agar containing nutrient fluid similar to that used for growing the cell layer. After solidification of the agar, the flasks are incubated for three days at 37°C. At this time they show round necrotic areas of 2 to 4 mm. in diameter, visible to the eye as bright areas against a dark background (Fig. 1). These areas are easily recognized under low-power magnification because the cells have been transformed into a granular debris that scatters the light. That these areas, which we call plaques, are produced by virus activity is shown by the fact that they are absent when the virus is absent and their number increases with the concentration of the virus. Prior treatment of the virus with specific antiserum reduces the number of plaques produced (table 1).

Each of these plaques is produced by one virus particle. This can be shown by determining the effect of dilution on the number of plaques produced by a given virus sample. The theoretical relation between the number of plaques and the virus concentration depends on the minimum number of virus particles required to produce a lesion. A linear dependence of the number of plaques on virus concentration can be obtained only if each plaque is produced by one virus particle. The results of several experiments show that this dependence is in fact linear, and so prove that one particle is sufficient to produce one plaque (table 2).

What fraction of the virus particles contained in a sample is able to produce plaques is not known. We have determined the relation between the number of particles able to produce a plaque under our conditions and the number of particles able to infect the chicken embryo by the standard inoculation procedure on the chorioallantoic membrane.[6] Two batches of 15 eggs each, incubated for 10 days, were inoculated with an amount of virus able to produce, respectively, 0.7 and 0.35 plaques on the average.

TABLE 3

COMPARISON OF THE TITER OF A SINGLE STOCK OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS BY PLAQUE COUNT AND EGG TITRATION

The plaque count titer is derived from Exp. 1, table 2 and equals 7.5×10^6/ml. The data of the egg titration are given here. Each egg was inoculated with 0.1 ml. of the virus dilution.

BATCH OF EGGS	VIRUS DILUTION	SURVIVING EGG FRACTION	$-\log_e$ SURVIVING EGG FRACTION	TITER, PER ML.
1	10^6	0.16	1.8	1.8×10^7
2	2×10^6	0.50	0.7	1.4×10^7

The eggs were then incubated for 30 hours at 37°C., then scored for live and dead. The live ones were further incubated for 24 hours to check their vitality; all survived. From the fraction of live eggs in each batch the average number of infecting virus particles with which each egg was inoculated was determined by assuming a Poisson distribution of the particles per egg. This assumption is legitimate in view of the proof previously given in this article that infection of the cells is produced by one virus particle. The data of one such experiment, reported in table 3, show that the titer was approximately twice the titer obtained by plaque count—a difference which is not significant because of the large standard error of egg titration. In another experiment the egg titration gave a titer somewhat lower than the plaque count.

We conclude from these experiments that there is a nearly one-to-one ratio between the number of plaques and the number of infective virus particles. This result on the one hand indicates that the plaque count is an

efficient assay technique; on the other hand, it establishes a basic concept concerning animal virus action, namely, that infection of an embryo is produced by one virus particle.

The use of the plaque count as an assay technique has important practical advantages, as shown by the fact that the accuracy obtained with only one flask of this type is equal to that obtained with not less than one hundred chicken embryos in the currently used Reed and Muench technique.

We do not know whether many other viruses will exhibit this property of producing plaques under our conditions. So far, we have tried only one other virus, the Newcastle Disease virus,[7] and the results here were positive. The plaques formed by Newcastle Disease virus and those formed by Western Equine Encephalomyelitis virus are distinguishable under the microscope; the areas of cell destruction in the former are outlined by a halo of abnormally large cells or cell clumps of unknown nature, whereas in the latter there is seen only cell debris. The possibility of distinguishing the plaques produced by two different viruses on the same flask should be very valuable for the study of problems where two different viruses are involved, as in the phenomenon of interference.

Summary.—Plaque formation by single virus particles has been obtained with the virus of Western Equine Encephalomyelitis in a monolayer of chicken embryo fibroblasts grown *in vitro*. That a plaque is produced by a single virus particle is shown by the proportionality between number of plaques and virus concentration. The comparison between the number of plaques produced and the fraction of chicken embryos infected by the same virus sample indicates that nearly all virus particles able to infect the embryo produce a plaque; there is therefore a nearly 1:1 relation between infecting particles and plaques.

Plaques produced by this virus are distinguishable from the plaques produced by one other virus tested, the Newcastle Disease virus.

[1] Obtained from Dr. S. Lennette, California State Department of Public Health, Berkeley, Calif.

[2] Shannon, J. E., Earle, W. R., and Walts, H. K., *J. Natl. Cancer Inst.* (in press).

[3] Earle, W. R., *J. Nat. Cancer Inst.* **4**, 165–212 (1943).

[4] Evans, V. J., Earle, W. R., Sanford, K. K., and Shannon, J. E., *Ibid.*, **11**, 907–927 (1951).

[5] Earle, W. R., *Ibid.*, **4**, 131–133 (1943).

[6] Beveridge, W. I. B., and Burnet, F. M., "The Cultivation of Viruses and Rickettsiae in the Chick Embryo," His Majesty's Stationery Office, London, 1946, p. 14.

[7] Strain B, obtained from F. B. Bang, Johns Hopkins Medical School, Baltimore, Md